Waiting in the wings: What can we learn about gene co-option from the diversification of butterfly wing patterns?

Chris D. Jiggins, Richard W. R. Wallbank and Joseph J. Hanly

Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ. UK

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Summary

A major challenge is to understand how conserved gene regulatory networks control the wonderful diversity of form that we see among animals and plants. Butterfly wing patterns are an excellent example of this diversity. Butterfly wings form as imaginal discs in the caterpillar and are constructed by a gene regulatory network, much of which is conserved across the holometabolous insects. Recent work in *Heliconius* butterflies takes advantage of genomic approaches and offers insights into how the diversification of wing patterns is overlaid onto this conserved network. WntA is a patterning morphogen that alters spatial information in the wing. Optix is a transcription factor that acts later in development to paint specific wing regions red. Both of these loci fit the paradigm of conserved protein coding loci with diverse regulatory elements and developmental roles, that have taken on novel derived functions in patterning wings. These discoveries offer insights into the 'Nymphalid Ground Plan' which offers a unifying hypothesis for pattern formation across nymphalid butterflies. These loci also represent 'hotspots' for morphological change that have been targeted repeatedly during evolution. Both convergent and divergent evolution of a great diversity of patterns is controlled by complex alleles at just a few genes. We suggest that evolutionary change has become focussed on one or a few genetic loci for two reasons. First, pre-existing complex *cis*-regulatory loci that already interact with potentially relevant transcription factors are more likely to acquire novel functions in wing patterning. Second, the shape of wing regulatory networks may constrain evolutionary change to one or a few loci. Overall, genomic approaches that have identified wing patterning loci in these butterflies offer broad insight into how gene regulatory networks evolve to produce diversity.

A major challenge in evolutionary biology is to understand how novel phenotypes can arise without disruption of existing gene function [1][Peichel, This issue; Soltis, this issue]. In general, organisms are well adapted to their current environment and subject to stabilising selection [2]. This is especially the case during development, which involves complex genetic interactions that need to be precisely coordinated to produce a functioning organism. The great majority of possible mutations that arise and alter developmental processes will tend to be detrimental. Moreover, there is only a relatively small set of proteins responsible for coordinating a huge variety of developmental processes, such as the canonical signalling pathways [3][Babonis and Martindale, this issue]. Changing the action of these pathways might occasionally lead to useful new patterns or structures, but would be far more likely to disrupt existing developmental processes.

One solution is provided by the '*cis*-regulatory hypothesis' [4–6]. *cis*-regulatory evolution provides a mechanism for proteins to adopt new functions through the evolution of novel regulatory interactions, which can be highly modular in their action. This was originally inspired by the widespread conservation of proteins, such as signalling molecules and transcription factors, despite their frequent re-deployment into different roles across the diversity of life. However, although generally acknowledged as a widespread mechanism for evolutionary change, there remain relatively few examples of recent evolutionary changes where the precise *cis*-regulatory interactions between genes involved are understood [5,7].

Heliconius wing patterns represent a recent phenotypic radiation with extensive diversification as well as convergence through the evolution of mimicry. This largely involves evolutionary diversification in gene regulation, providing a promising system for dissecting the process of *cis*-regulatory evolution [8,9]. The patterns have arisen recently enough that it is feasible to identify the exact DNA changes that produce different patterns. However, the diversity is also old enough to represent complex novel phenotypes that have undergone a history of repeated natural selection [10]. The rapid evolutionary changes therefore lie somewhere in between recent microevolutionary changes occurring over a few generations, such as the famous melanic peppered moths, and the macroevolutionary patterns of diversification seen for example in the diverse body plans of the arthropods. As such, *Heliconius* patterns offer an opportunity to understand how changes in morphology are fine-tuned by natural selection. The developmental context for this diversity is the early formation of the insect wing. Although little is known about this process in butterflies, we can infer a great deal from other insects, notably *Drosophila*.

Early wing development in the fly

In *Drosophila* there is a rich landscape of spatial information across the developing wing, in the form of localised expression of genes, established from the first instar of larval development. For example, *engrailed* is expressed in the posterior compartment of the wing and *cubitus interruptus* in the anterior compartment [11,12]. These indirectly activate *decapentaplegic* between the two compartments, establishing a gradient moving out from a central stripe in the wing [13]. Similarly, dorsal and ventral wing surfaces are defined by expression of *apterous* and *vestigial* respectively in the second instar [14,15]. This is just part of a complex network of interacting genes that establishes spatial information. Many of these genes and their expression patterns are known to be conserved between flies and butterflies. Ultrabithorax, Engrailed, Cubitus Interruptus and Apterous are all seen in butterflies in patterns very similar to their known role in flies [16–20]. It seems likely therefore that early events in the development of the wing have been conserved throughout the evolution of holometabolous insects, over some 300 million years of evolution [21].

Different scale pigments and ultrastructures produce wing patterns

Overlaid onto the conserved wing structure are pixelated coloured scales. *Heliconius* colours are mainly derived from chemical pigments. Thus, the red, orange and brown colours are ommochrome pigments whilst the yellow colour is 3-hydroxykynurenine (3OHK), a biochemical precursor of the red pigments [22]. Black patches are pigmented with melanin [23]. Expression of pigmentation enzymes is highly coordinated across the wing and in different scale types. *kynurenine formamidase*, *ebony* and *cinnabar* are upregulated in red patches and *tan* in black regions, in accordance with their known function [24–26]. These expression patterns are repeatable across wing regions and species, suggesting highly modular gene regulation. White, green and blue colours are caused by scale ultrastructure, but pigmented colours are also associated with specific scale ultrastructures [27–29]. The yellow/white (Type I), black (Type II) and red/orange (Type III) scales differ in the spacing and frequency of the ridges and cross-ribs. The timing of scale cell enlargement and maturation also differs between scale types, with red and yellow/white-fated cells becoming mature earlier than the black-fated cells, and evident as opaque regions in the wing [25,29]. It seems likely that these scale structures have evolved to optimise appearance, and likely enhance brightness and hue of coloured patches.

These are the two ends of the development of a wing. At the beginning, a highly conserved set of patterning factors establish spatial information in the wing – so highly conserved that expression patterns in a butterfly are similar to those in *Drosophila*. At the end of the process, there are complex differences in spatial arrangement of scale colour and structure, even between closely related populations, associated with changes in gene expression and the timing of scale development. So what happens in the middle? How is the conserved spatial information translated into the diversity of butterfly wing patterns?

The pattern locus and Wnt signalling

Early crossing and mapping experiments revealed that a single locus, known as *Ac* in *H. melpomene* and *Sd* in *H. erato*, controls various aspects of the shape of forewing band patterns across natural populations [30–32](Figure 1). *WntA* was identified as the functional gene at this locus through a combination of genetic linkage mapping using RAD-seq, a population genetic signal of divergence, and differences in expression patterns in developing wings [33,32,34,35]. *WntA* is expressed in the final larval instar where it shows diverse expression patterns associated with black regions in the centre of the forewing (Figure 2). Additional evidence for the role of *WntA* comes from heparin injection into wing tissue of developing *Heliconius* pupae, which leads to changes in adult wing pattern comparable to genetic effects of *Ac* [33,34]. Heparin binds Wnt family ligands and promotes their mobility through tissue, and although its effects are not specific to WntA, the combination of genetic mapping, morphogen experiments and expression studies builds a compelling case for the role of *WntA* in forewing patterning.

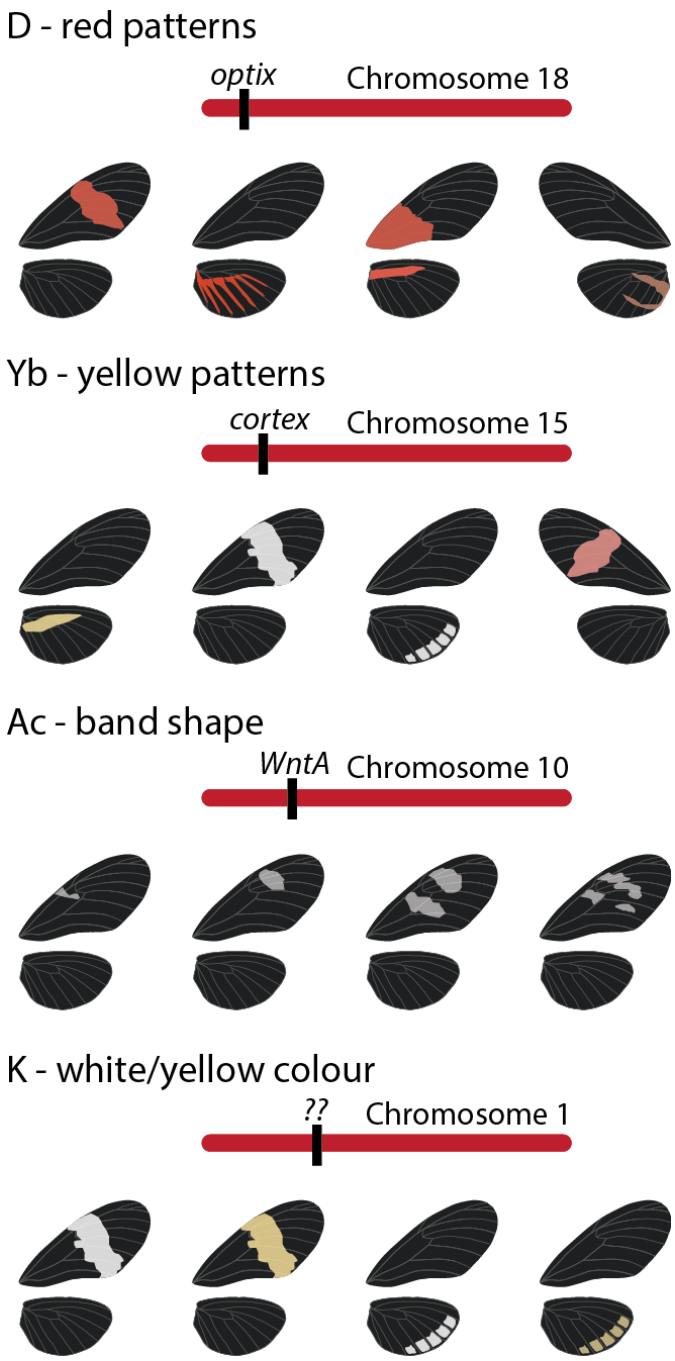

Figure 1 Summary of major wing patterning genes in *Heliconius.*
Four major loci control most of the phenotypic variation in *Heliconius* patterns. All wings indicate dorsal patterning apart from two wings in reverse orientation indicating ventral patterns. Red patterns are controlled by *optix*, a transcription factor, yellow/white patterns by *cortex*, and forewing band shape by *WntA*. The pink ventral pattern indicated for the *cortex* locus is the presence of white scales in the red forewing band underside, giving a pink appearance (*Vf* locus).

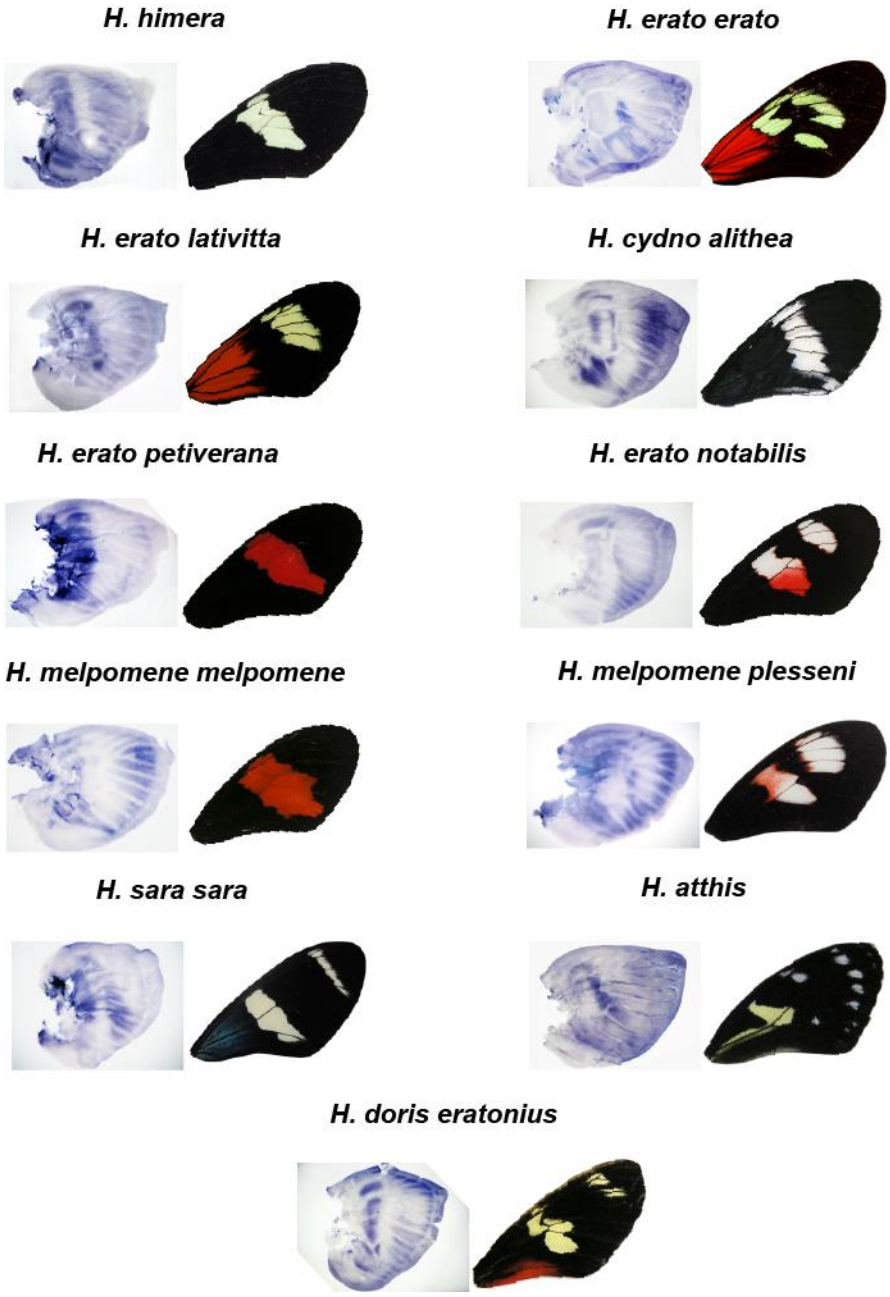

Figure 2 Expression patterns of *WntA* in larval wing discs of *Heliconius* species
WntA is expressed in larval wing discs in regions that are fated to become black in the adult butterfly. Note that not all black regions show *WntA* expression. Images courtesy of Arnaud Martin [33].

WntA is also associated with adaptive pattern variation in *Limenitis arthemis* [34]. *Limenitis* and *Heliconius* diverged about 65MY ago, so the involvement of *WntA* in patterning implies a shared and relatively ancient role for this gene in nymphalid wing pattern specification. It seems likely that the *wingless* signalling pathway has a role in wing pattern establishment that is shared across all the Lepidoptera [16,36], but it remains unclear at which point the *WntA* gene was specifically co-opted into pattern specification.

It is not yet clear how *WntA* functions in pattern specification, but from analogy with its paralog *wingless*, it seems possible that it acts as a morphogen to establish patterned regions of the wing. It has long been predicted that the evolution of patterns on animals might involve changes in morphogens [37]. However, to date there are surprisingly few examples of evolutionary change in which the morphogen is the target of selection. One potential example is the characteristic spotting pattern in *Drosophila guttifera,* which is correlated with *cis*-regulatory variation controlling expression of *wingless* [38]. In a similar way, *cis*-regulatory changes at *WntA* alter pattern specification in *Heliconius*. It is therefore an intriguing possibility that *WntA* might represent an example of evolutionary change in the regulatory control of a morphogen. Nonetheless, it is perhaps more common for recent developmental evolution to involve genetic changes at transcription factors, which interpret established patterning. *Heliconius* also provide a compelling example of this process.

The red gene optix

Wherever populations and species differ at red patterns, such as forewing bands or hindwing rays and bars (Figure 1), crossing experiments have shown Mendelian inheritance controlled by a single major effect locus located on chromosome 18 (the D locus, also B, R and Y) [39]. Again, a combination of linkage mapping followed by positional cloning, expression studies and population genomics, has identified the transcription factor *optix* as the functional locus [40]. In contrast to *WntA*, *optix* acts later in development to paint colours onto the existing spatial patterns on the wing (Figure 3). A region of non-coding DNA some 100kb to the 3' end of *optix* is most closely associated with wing patterns in natural populations [10,41,42], and there is a precise correspondence between *optix* expression in the developing pupal wings and red patches on the adult, confirmed by both *in situ* hybridisation and immunohistochemistry [40,43]. Pattern forms show no consistent differences in protein coding sequence at *optix*, nor do backcrosses reveal major differences in *trans*-acting factors, suggesting the differences are again *cis*-regulatory [40]. This regulatory locus therefore encodes precise instructions that turn on *optix* in specific regions of the wing, determining their fate as type III red pigmented scales.

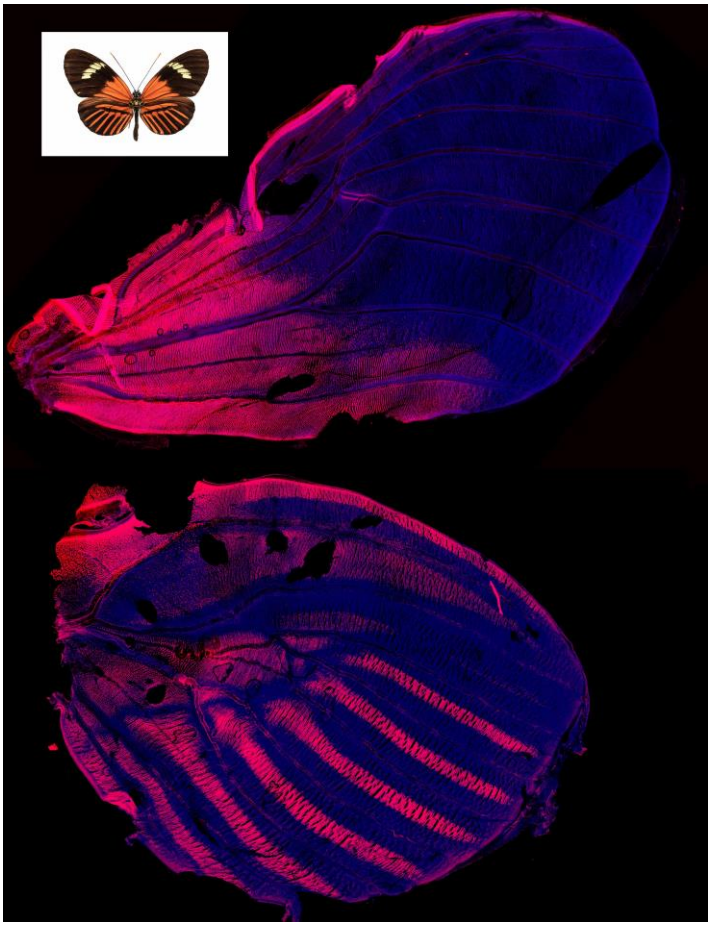

Figure 3 Antibody stain against Optix in pupal wings
The *optix* gene is expressed during pupal wing development in regions that show perfect concordance with red patches in the adult wing. This is an individual of *H. elevatus pseudocupidineus*.

In the fruit fly, Optix is involved in a wide range of developmental processes including eye and wing development [44,45], and the same is likely true in *Heliconius*. The ancestral function of the Six gene family, to which *optix* belongs, is in specification of the forebrain and so a role in the eye may represent a retention of part of this ancestral function [46]. It is therefore unsurprising that the coding sequence of this gene is highly conserved across the insects. *optix* has therefore adopted its new function in scale specification both through *cis*-regulatory evolution, leading to its expression in wing scale precursor cells, but also by adopting new downstream targets among genes involved in pigmentation and scale structure. It has been suggested that co-option of *optix* may have occurred from an ancestral role in eye pigmentation [47,48], although the expression of *optix* in the fly wing perhaps makes it more likely that there was co-option from an ancestral role in wing development.

In addition to red scales, *optix* is also associated with development of spear-shaped 'wing coupling' scales that lie in the region of overlap between fore- and hindwing in many numphalid butterflies [43]. More specifically, in two related species *Agraulis vanillae* and *Dryas iulia, optix* is associated with the specification of a small group of cells found in males, and associated with pheromone-producing scales [43]. In summary, *optix* has adopted a variety of functions related to scale-specification and represents an excellent example of a conserved protein-coding gene that has been co-opted into a novel function through regulatory evolution.

The origins of recent novelty through enhancer shuffling

Much of the evolutionary novelty at *optix* must involve altering the *cis*-regulatory interactions of this locus with the patterning landscape of the wing. However, genomic data has also confirmed another mechanism which can potentially provide novelty on a shorter timescale. It has long been speculated that hybridisation and recombination could play a role in *Heliconius* diversification [49]. Using a large set of genome sequences, haplotypes associated with specific pattern elements, known as dennis and ray patterns, were identified in *H. melpomene* and its relatives [10]. This analysis took advantage of phenotypes possessing these elements alone, including the Guiana race *H. melpomene meriana* with dennis but not ray and *H. timareta timareta f. contigua* from Ecuador with ray but not dennis. Analysis of the sequences of these populations permitted identification of small regions of non-coding DNA just a few kilobases in length that are consistently associated with specific patterns. These regions are presumed to act as modular enhancers, turning on *optix* in specific regions of the developing wing, although we currently cannot rule out the possibility that some act instead to repress specific regions of expression. By shuffling these modules into new combinations within and between species, introgression and subsequent recombination has generated novel patterns without the need for the evolution of complex novel regulatory machinery [10]. Thus introgression and recombination complements regulatory evolution as a mechanism for generating evolutionary novelty.

The yellow locus

The third major patterning locus controls the placement of yellow pattern elements (known as *Yb* in *H. melpomene*) and was the first to be mapped using genetic markers [50]. Genetic associations in wild populations are diffuse and there are no clear blocks of genetic sequence repeatedly associated with specific patterns as seen at *optix*. Nonetheless, there is a strong peak of association around a gene, *cortex*, which also shows differences in expression between wing patterning forms [51]. Intriguingly, this locus also controls adaptive variation in the peppered moth, *Biston betularia*, the famous case of adaptation to darkened soot-covered tree trunks of Industrial Revolution Britain [52]. *cortex* belongs to the *fizzy* family of cell cycle regulators, which act to control the cell cycle through the action of the anaphase-promoting-complex. *cortex* itself is expressed in the female germline in *Drosophila* and is essential for the completion of meiosis in oocytes [53]. However the lepidopteran gene has evolved rapidly and is highly divergent from its orthologue in *Drosophila*, so may have adopted an entirely novel function [51]. *cortex* might play a role in regulating the heterochrony of scale development associated with different coloured scales, perhaps during the polyploidisation that occurs during scale maturation. Finally, there are other nearby genes that may also play a role in pattern specification, notably *domeless*, a receptor molecular in the JAK/STAT signalling pathway [54]. However, it is clear that this locus represents another ancient patterning gene that is co-opted repeatedly into the control of evolutionary novelty.

Revisiting the nymphalid ground-plan

The discovery of these patterning genes in *Heliconius* can offer insights into long-standing questions regarding the evolution of pattern diversity. Across the nymphalid butterflies, it has been proposed that there is a ‘ground-plan’ that underlies wing patterning (Figure 4) [23,55–58]. Many nymphalid patterns are composed of repeated elements often associated with wing venation, and there are clear homologies between such elements across many nymphalid lineages. Many of these elements form part of ‘symmetry systems’ of pattern elements with paired symmetry. The simplest of these are parallel lines crossing the wing anteroposteriorly, which are hypothesised to have formed around morphogen sources. The symmetry inherent in

many of these elements is therefore thought to result from diffusion of a morphogen from a central source, producing symmetrical patterns around that source.

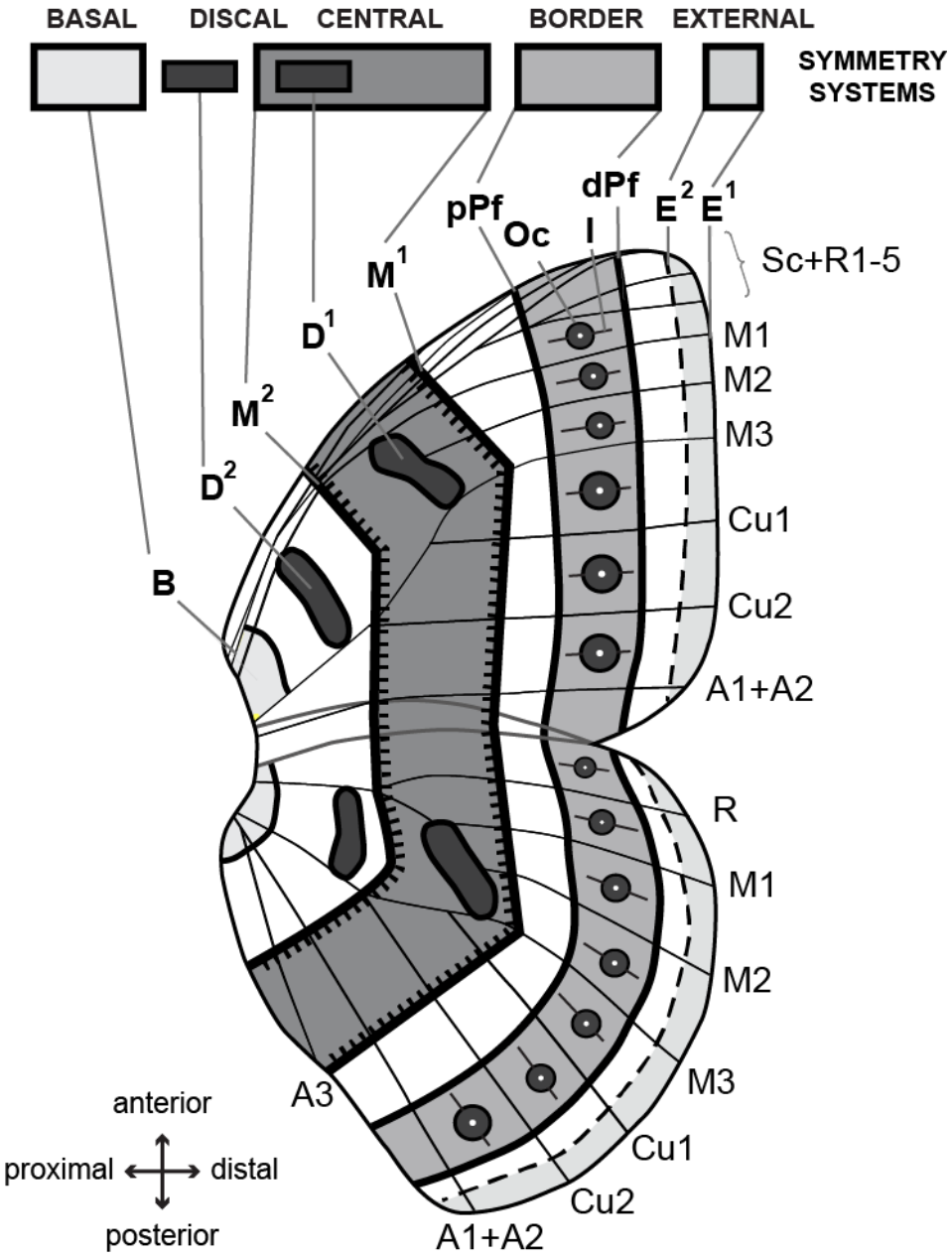

Figure 4 The Nymphalid Ground Plan
The Nymphalid Ground Plan with nomenclature derived from [57]. Image courtesy of Arnaud Martin [59]. Elements are Basal (B), Discal (D^1 and D^2), Medial (M^1 and M^2), proximal and distal parafocal (pPf and dPf), Ocelli or eyespots (Oc), Intervein (I), and External (E^1 and E^2). Wing veins are labelled to the right using standard nomenclature.

Following the discovery of Wnt signalling in the patterning of *Heliconius* wings, it is becoming possible to test these homologies using genetic information. For example, in wing discs dissected from fifth instar larvae of *Euphydryas chalcedona*, expression of *Wnt* genes marks the development of the basal (*WntA*), discal (*Wnt1/Wnt6/Wnt10*), central (*WntA*), and external (*WntA*) symmetry systems [59]. These same genes are found to be associated with pattern

elements in other species including *Vanessa cardui* and *Agraulis vanillae*, which confirms homology between putative ground-plan elements across these species, and supports the Nymphalid Ground Plan (NGP) hypothesis for homology more widely across the nymphalid butterflies [59].

Our current appreciation of the NGP owes a great deal to the work of Nijhout, who formulated our current understanding of the elements. Whilst acknowledging our debt to this work, we suggest some areas in which the NGP can be updated. Notably, Nijhout delineated different wing regions as either 'pattern' or 'background': '*In studying pattern formation it is essential to distinguish between the pattern and the background upon which the pattern develops'* [55] and '*The majority of pattern elements consist of dark-coloured shapes on a lighter background*' [56]. However, the expression and inheritance patterns of *optix* are hard to reconcile with this dichotomy between pattern and background. Yellow is typically considered as background, with black portions of the wing foreground, and the shapes of these black patterns are modulated by expression of *WntA* and *cortex*. Consistent with this, in *H. erato* the forewing band can be either red or yellow; expression of *optix* turns on red in an otherwise yellow forewing band region. However, in *H. melpomene,* hybrids between Postman and Amazonian forms, the hindwing yellow bar "overprints" the hindwing portion of dennis [31], which should not be possible if yellow constitutes the background. Also, red patterns appear as both modifying the background yellow and foreground black patterns. Alternatively, we could consider black to be the background and red/yellow to be the pattern, as has been typical in the evolutionary genetics literature [30]. Consistent with this, in the hindwing of both *H. melpomene* and *H. erato*, and in the *H. melpomene* forewing, *optix* places red elements onto an otherwise black wing. However, NGP homologies among the wing patterns of other butterflies are less clear in this hypothesis. Additionally, WntA expression patterns described above do not agree with yellow as pattern. Neither hypothesis is consistent with the fact that *optix* sometimes controls red patches that correspond to 'background', while in other cases it controls red patches that are 'pattern', nor that sometimes red overprints yellow, yet sometimes yellow overprints red [55]. Instead, we suggest that the distinction is not meaningful: there is a hierarchy of factors that interact by both activation and inhibition of downstream factors. Optix is a paintbrush that can be co-opted to colour wing regions already outlined by any part of this upstream patterning landscape.

It has also been argued that NGP elements develop independently in each wing cell, with wing veins acting as landmarks during development [56]. However, *WntA* expression domains are clearly part of a wing-wide patterning system, such as the front-back white stripe of *Limenitis* [34,59]. Vein information can modulate this underlying whole-wing system to varying degrees, such as repeated patterns of the external symmetry system, where the focal scale cells of eyespots or chevrons are defined by veins. However, a vein-less *Heliconius* mutant suggests that there is little influence of wing veins on pattern elements in this group [60]. Similarly, a vein-less mutant of *Papilio xuthus*, which shows an intact pattern but lacks vein-specific modification, demonstrates how wing-wide patterns can be altered by vein-associated information [61].

We therefore suggest that the NGP does not represent a series of vein-dependent repeated elements. Rather, it is a whole-wing patterning system derived from a conserved set of genes that control early stages of wing differentiation. Wing veins and wing pattern are two outcomes of spatial patterning across the wing. The process of wing patterning (as indeed is the case for all developmental processes) is a hierarchy of increasing complexity in a gene regulatory network (GRN), which moves from simple whole-wing spatial information established early in development, through to ever more complex spatial information later in development (Figure 3) that includes, but is not limited to, wing venation. Downstream wing patterning factors, such as

optix, can tap into different levels of this hierarchy in an *ad hoc* manner as required by the demands of evolution. So rather than wing patterning being seen as solely the outcome of a set of homologous vein-dependent elements, we should envisage a GRN of increasing complexity, with different levels of information available for patterning (Figure 5).

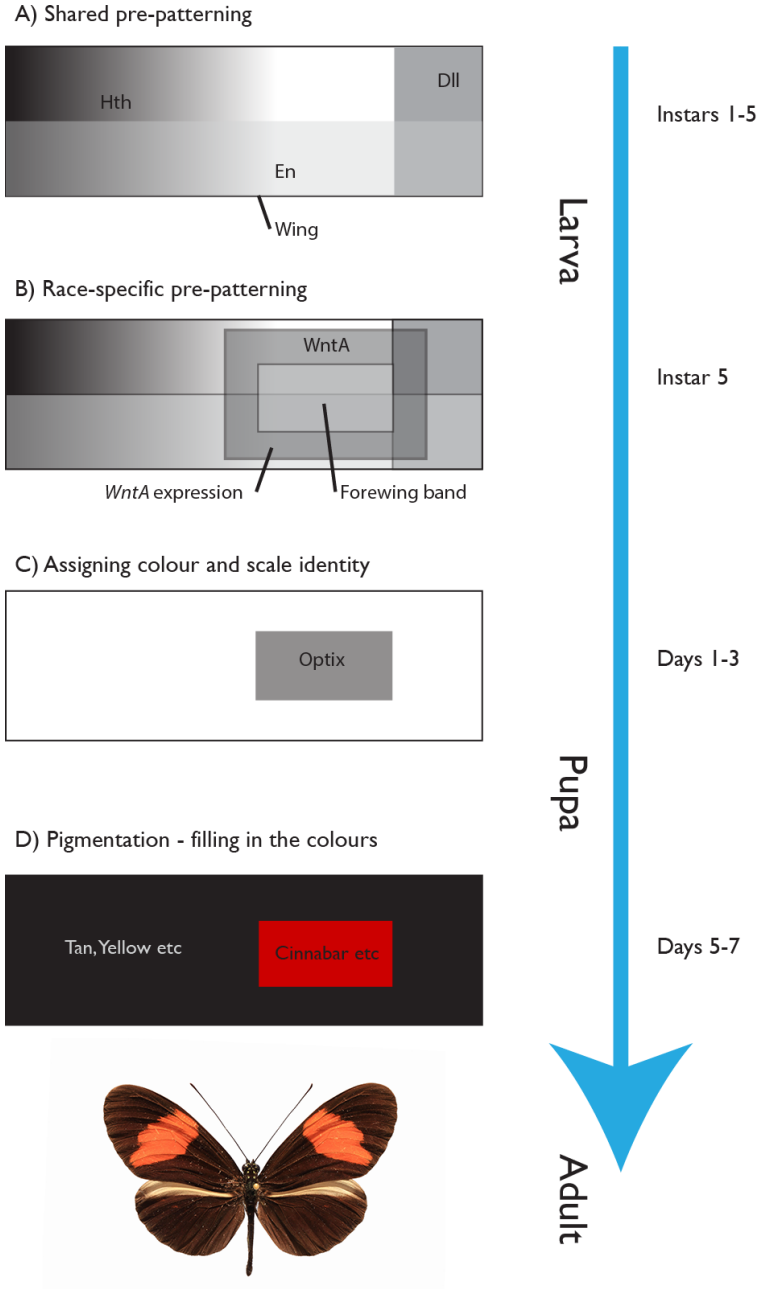

Figure 5 Summary of wing pattern development in *Heliconius*.
A schematic wing is shown during larval and pupal development. A) Early in larval development, many of the genes involved in early wing patterning are shared across the insects and therefore highly conserved in their expression domains. Putative patterning factors inferred from *Drosophila* include Homothorax (Hth – proximal gradient), Engrailed (En – posterior) and Distal-less (Dll - distal). B) Late larval stages start to differentiate in gene expression patterns between different wing pattern forms within *Heliconius*, such as at *WntA* which patterns some of the regions fated to be black and defines the forewing band. C) During pupal stages, early patterning information is interpreted into different colours – notably *optix* which defines presumptive red wing regions, such as the red forewing band already pre-patterned by *WntA*. D)

Finally, in the second half of pupation, the expression of patterning genes such as *optix* is converted into colours via localised expression of pigment synthesis enzymes such as *cinnabar* (red) and *tan* (black). Note the regulatory links between all of these stages are largely unknown. Ecdysone receptors may be involved in regulating the timing of pattern formation.

Why hotspot genes?

One of the surprising aspects of *Heliconius* wing pattern evolution is the repeated involvement of the same few genes in pattern evolution (Figure 1). It is now clear that, to a fairly remarkable degree, convergent evolution involves utilisation of a small handful of genetic loci. The clearest example is provided by the Amazonian dennis-ray races of *Heliconius melpomene* and *H. erato*. Sequence data from the *optix* locus shows that within each of these lineages these patterns have arisen independently, most likely from a red-banded postman ancestor [10,42,62]. Genetic change at the *optix* locus underlies the independent evolution of similar phenotypes in at least two lineages.

However, genetic 'hotspots' for wing pattern evolution represent more than just the same pattern arising multiple times. The 'hotspot' genes are co-opted not just for convergence but also for novel evolution of a diversity of different patterns [50,63]. For example, *WntA* (the *Ac* locus), generates double bands in the co-mimics *H. m. plesseni* and *H. e. notabilis*, single bands in *H. cydno* and many races of *H. melpomene* and *H. erato*, spotted yellow forewing patterns in Amazonian races of the same species, and small yellow spots in *H. hecale* and *H. ismenius* that mimic ithomiine species [33,32,35,59]. Similarly, it has been used to remove white bands when distantly related *Limenitis arthemis* mimics *Battus philenor* [34]. Thus, a single gene underlies evolution of a huge variety of adaptive patterns that generate mimicry and diversity. In the terminology of Martin and Orgogozo, this is both an *inter-lineage* and *intra-lineage* hotspot for evolution [64]. Since hundreds of genes are likely involved in development of a wing, why are only a few involved in pattern evolution? A wide variety of possible reasons for hotspots have been proposed in the literature [7,65–67] – here we discuss two that seem most relevant to *Heliconius*.

Complexity of cis*-regulatory elements*

As *cis*-regulatory control at a particular genomic region increases in complexity, the chances of further interactions being accrued at that locus may become more likely through positive feedback effects. For example, a region of the genome with existing wing-specific *cis*-regulatory elements is more likely to be predisposed to adopt new wing-related functions. If binding sites are already present for stage and tissue-specific transcription factors, it will be much more likely for a new function to be adopted in that particular tissue. In the case of *optix*, for example, in order for expression to be specific to hindwings or forewings and to show spatial localisation across the wing, it needs to be driven in the wing scale precursor cells during early pupal development. *optix* is expressed in the wings of *Drosophila*, although its function is not known [7,62–64]. At some point, *optix* must have come under the control of factors that define spatial information in the wing and specific to scale precursor cells. In addition, its role in scale specification implies that the Optix protein evolved downstream regulatory links that allowed it to control scale morphology, presumably through regulating genes that control actin polymerisation and thereby scale structure [68]. In the case of red patterns this also includes direct or indirect regulation of pigmentation genes, including *cinnabar*, *ebony* and *tan*. Therefore, once *optix* had evolved one of its functions in scale specification, it would have been pre-adapted to assume new and associated functions. This eventually led to the related roles of defining wing coupling scales, red pattern elements and sex-specific scales in the *Agraulis*/*Dryas* group [43].

When selection favoured a new mode of pattern specification, *optix* was already waiting in the wings ready to fulfil this role.

All of these functions require complex inputs to be interpreted correctly. From previous *Drosophila* and lepidopteran studies, we can predict that the regulatory region of *optix* directly or indirectly receives input from Ultrabithorax (distinguishing hind- and forewing), Achaete-Scute (defining the scale precursor cells), and Engrailed (distinguishing the anterior/posterior compartment), to name a few candidates. The acquisition of a new interaction that produces a novel domain of *optix* expression does not, therefore, require a large number of steps, as compared to complete *de novo* evolution of a new wing regulatory element at a different gene. Similar arguments have been made to explain repeated *cis*-regulatory evolution of the same enhancer element at the *yellow* gene in placing melanic spots onto the wings of fruit flies [66].

Multi-function developmental genes such as Hox genes commonly have large and complex regulatory regions that are the outcome of many millions of years of repeated regulatory evolution targeted at the same locus [69,70]. Similarly, both WntA and optix are adjacent to large non-coding regions that likely encode multiple regulatory functions. These broad regulatory regions may encode many separate modules. It seems likely that this highly modular nature further allows them to recombine into new combinations, or adopt new tissue-specific functions, facilitating evolutionary diversification.

The hourglass shape of networks

In addition to the complexity of *cis*-regulatory control, particular positions in a GRN may be especially prone to evolutionary change [7,67]. For example, mutations in genes that act earlier in a GRN are more likely to generate a phenotypic effect than later genes, due to their greater number of downstream dependencies. For that phenotypic change to be viable, however, the mutation must produce a coherent outcome that is favoured by selection, but also avoid detrimental side-effects (i.e. deleterious pleiotropy). An example of such a gene is *shaven-baby,* associated with loss of larval trichomes in multiple *Drosophila* species [71,72]. The expression of *svb* in a cell is enough to induce development of a trichome structure from a precursor cell. In this way, the regulatory region of *svb* integrates information from many upstream genes to determine whether expression should be induced and therefore which cells will make trichomes. This triggers a signalling cascade that is relayed to many downstream genes that are involved in making a trichome. Hence, the larval GRN includes a trichome 'module' that is controlled by *svb*.

The GRN is therefore hourglass shaped, with the regulatory region of *svb* at the centre acting as a focus for multiple upstream patterning factors, whilst the Svb protein stimulates an expanding cascade of interactions downstream, representing the trichome module. The consequences of this GRN shape are that the phenotypic effects of mutations depend on where in the hourglass they occur. The cohort of patterning factors that provide *svb* with regulatory information are also likely to pattern other developmental processes. Modifying *svb* expression through mutation of upstream factors will potentially have deleterious effects on multiple aspects of development. Alternatively, mutations downstream of *svb* will affect the cascade of genes that work in concert to construct every trichome in the larva. Therefore, mutations in genes both upstream and downstream in the *svb* GRN will lack the subtle specificity and control of patterning required to generate a new and beneficial trichome phenotype. In summary, there is effectively only one way to make new trichome patterns, by altering the spatial localisation of *svb* through *cis*-regulatory evolution. Through positive feedback, the *svb* regulatory region becomes a locus of major effect as functional mutations accumulate and regulatory complexity increases. It seems likely that

genes such as *optix* and *WntA* act in a similar way to *svb* and form what are known as 'input-output genes' at the centre of the hourglass GRN for scale development. This remains speculative, as work is still underway to elucidate the regulatory network that controls patterns, but will become testable over the coming years.

Is there a reason why some GRNs have evolved such an hourglass shape, with distinct regulatory modules controlling particular structures [73]? The answer may lie in the need to coordinate the development of repeated structures. Larval trichomes on the fly, and scales on a butterfly wing, all have in common a repeated structure involving a complex developmental trajectory. This requires the coordinated expression of many genes in the right sequence within each scale or trichome precursor cell. The output of a system for regulating the development of such a repeated structure therefore needs to be under rigid binary control, whilst tight coordination of downstream events during development will be favoured in order to ensure a strongly canalised output. It seems plausible that highly repeated structures might favour the evolution of regulatory modules and an hourglass network shape through a process of network clustering [73].

In *Heliconius* wing patterns, the development of scale structure and colour is highly coordinated [28]. Furthermore, this coordination is stronger in coloured regions of the wing as compared to regions in the same individual that are less visible [29]. Perhaps selection for visual signalling to predators might have favoured the evolution of tight coordination of scale development and pigmentation in order to produce highly contrasting coloured patches that more effectively signal distastefulness. Once such a network structure has evolved then evolutionary change in the wing patterns will become focused on the single input-output gene at the centre of the network. Although an hourglass-shaped network constrains evolution through restricting change to a particular locus, this network structure might also facilitate diversification as only changes to the expression of a single gene are required to produce a new scale type.

In summary, both the shape of gene networks and the accumulation of complex regulatory regions can contribute to biasing evolutionary change towards a particular locus. Much of this is speculative given limited current knowledge of the *Heliconius* patterning networks, but will be tested empirically over the coming years. Specifically, we predict that 1) the regulatory locus of *optix* integrates information from many input signals in order to generate spatially localised *optix* expression patterns; 2) the same locus regulates many downstream targets, via Optix. This may involve direct or indirect control of actins, pigmentation enzymes etc., but importantly there are many downstream targets. If Optix were to regulate just a single downstream gene that in turn upregulated red scale production, then this would not support the hourglass hypothesis. Similarly, if the production of red scales went through several bottlenecks and other genes up- and downstream of *optix* were also sufficient for red scale determination, then this would cast doubt on the uniqueness of *optix* as the sole viable target for the evolution of this phenotype.

Conclusions

Recent advances in genomic techniques have permitted population genomic and linkage mapping studies and led to the identification of molecular mechanisms underlying much of the *Heliconius* radiation. While *Heliconius* lack many of the genetic tools available in *Drosophila*, for example, they nonetheless possess a great diversity of evolutionarily recent allelic variation, which can offer insights into how and why particular genes are targeted by natural selection. In the future, we will need direct experimental tests of the action of major patterning genes. Which mutations are critical for making a novel phenotype, and how many mutations are involved in generating each new phenotype? What are the upstream and downstream regulatory links and how do major patterning loci interact with one another? Is *optix* expression alone sufficient to produce novel

wing pattern phenotypes? These questions will be answered once we have tractable genome editing tools working in *Heliconius* and can manipulate individual sequences to test specific hypotheses. Most exciting would be to move specific regulatory elements between different genetic backgrounds in order to test the specificity of their action in producing novel wing patterns. The ultimate goal is to set up an experimental system in which the influence of single mutational changes on patterning could be investigated.

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Figure Legends

Figure 1 Summary of major wing patterning genes in *Heliconius.*
Four major loci control most of the phenotypic variation in *Heliconius* patterns. All wings indicate dorsal patterning apart from two wings in reverse orientation indicating ventral patterns. Red patterns are controlled by *optix*, a transcription factor, yellow/white patterns by *cortex*, and forewing band shape by *WntA*. The pink ventral pattern indicated for the *cortex* locus is the presence of white scales in the red forewing band underside, giving a pink appearance (*Vf* locus).

Figure 2 Expression patterns of *WntA* in larval wing discs of *Heliconius* species
WntA is expressed in larval wing discs in regions that are fated to become black in the adult butterfly. Note that not all black regions show *WntA* expression. Images courtesy of Arnaud Martin [33].

Figure 3 Antibody stain against Optix in pupal wings
The *optix* gene is expressed during pupal wing development in regions that show perfect concordance with red patches in the adult wing. This is an individual of *H. elevatus pseudocupidineus*.

Figure 4 The Nymphalid Ground Plan
The Nymphalid Ground Plan with nomenclature derived from [57]. Image courtesy of Arnaud Martin [59].

Figure 5 Summary of wing pattern development in *Heliconius*.
A) Early in larval development, many of the genes involved in early wing patterning are shared across the insects and therefore highly conserved in their expression domains. B) Late larval stages start to differentiate in gene expression patterns between different wing pattern forms within *Heliconius*, such as at *WntA*. C) During pupal stages, early patterning information is interpreted into different colours – notably *optix* which defines presumptive red wing regions. D) Finally, in the second half of pupation, the expression of patterning genes such as *optix* is converted into colours via localised expression of pigment synthesis enzymes such as *cinnabar* (red) and *tan* (black).